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The diagram illustrates a laser scanning system. At the top, a laser beam (52) is reflected by a mirror (58) and passes through an external frequency doubler (EXT. FREQ. DOUBLER) to produce a 2 MHz signal. This signal is fed into an oscillator driver (OD DRIV) (54), which then drives a laser diode (DL) (51). The laser beam (52) is directed by a mirror (59) through a series of lenses (60, 61, 62, 63) and a scanning mirror (64) to a drum (200). The drum is coated with a photoconductor (POLY CHROM) (65) and has a reference detector (REF DET) (66) and a photodiode (67) for feedback. The drum is also connected to a phospor array (72) and an image intensifier (71). The phospor array is connected to an array detector (73), which is further connected to an A-D converter (700). The A-D converter is connected to a data value acquisition & filter (702), which is connected to a data processing unit (808). The data processing unit is connected to a display (812(b)) and a printer (812(a)). The system also includes a frequency synthesizer (FREQ. SYNT.) (57) and a phase-locked loop (PLL) (56) that generates a 38 MHz signal (55) for a modulator driver (ML DRIV) (53). The modulator driver is connected to the laser diode (DL) (51). The system is powered by a 200V source (200) and a 2 MHz signal (52).

A digital frequency domain fluorometer utilizing a computer-controlled digital data acquisition system is used for the study of fluorescence and phosphorescence phenomena in the bio-chemical, biological and bio-physics arts. The computer (808) is used for the direct collection of data, as well as for the filtering and calculation of the phase and modulation values of the sample under study. From these values, fluorescence lifetimes can be determined. The digital data acquisition system (702 and 808) provides for the simultaneous collection and processing of several modulation frequencies. In addition, the digital frequency domain fluorometer can utilize an array detector (72) for detecting the modulated light from the various samples under study. The use of the array detector (72) provides a means for independently collecting data over a large number of pixels. This configuration allows for a time resolved image to be collected at once.

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METHOD AND MEANS FOR  
PARALLEL FREQUENCY ACQUISITION IN  
FREQUENCY DOMAIN FLUOROMETRY

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GOVERNMENT SUPPORT

The invention described herein was made in connection with work performed under a grant or award from the Division of Research Resources of the National Institute of Health.

BACKGROUND OF THE INVENTION1. Field of the Invention

The present invention relates to the field of analytical chemistry, and particularly to the study of fluorescence and phosphorescence phenomena in the biochemical, biological and biophysical arts.

2. Discussion of the Prior Art

The use of fluorescence spectroscopy for the study of the dynamics of macromolecules is becoming more widespread as more sophisticated instrumentation is being developed. Although fluorescence spectroscopy has developed into a widely accepted technique in the physical and chemical sciences as well as in the biological sciences, the practical utility of fluorescence methods is still limited by the availability of fluorescence spectroscopy instrumentation capable of measuring such events accurately.

Fluorescence is the rapid decay from a higher to a lower state of the same multiplicity. The natural time window of fluorescence is suitable to resolve dynamic events occurring in the nanosecond (ns) to pico-second (ps) time region. The above characteristics, coupled with the sensitivity of the excited state of a fluorophore to the

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physicochemical properties of its environment, is a major  
reason why fluorescence spectroscopy techniques are so  
1 frequently used in the study of micro-biological structures  
and functions.

The greatest interest is in measuring dynamic  
events displayed in the kinetics of intensity decay  
5 (fluorescence lifetimes) and anisotropy decay. The  
fluorescence lifetime reflects not only the intrinsic  
radiative rate of the excited state, but also the  
interactions of the fluorophore with the environment.  
Anisotropy decay measures the displacement of the emission  
10 transition dipole with time after excitation and thus  
reflects the rotational motion of the fluorophore. The rate  
and the amplitude of the rotational motion in a given time  
are themselves dependent on the free volume, the microscopic  
viscosity of the local environment and the forces acting on  
15 the excited molecule. Therefore, anisotropy decay indirectly  
describes the structure and dynamics of the fluorophore's  
environment. Clearly, a detailed study of the fundamental  
fluorescence observables (spectrum, quantum yield, lifetime  
and anisotropy) can provide substantial information about a  
20 biological macromolecule and its surrounding. Additional  
insight can be gained, if the system is physically or  
chemically perturbed, for example, by temperature or  
viscosity change or the presence of fluorescence quenching  
agents. The frequently complex fluorescence signal from  
25 biological systems does not easily yield to mathematical  
analysis and it may be difficult to correlate a physical  
event with the result of the analysis.

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The time decay of fluorescence is usually measured using one of two accepted, but different approaches.

1 Measurements of fluorescence decay can be made in the time domain using the popular technique of correlated single photon counting (SPC), or in the frequency domain by  
5 determining the phase delay and the relative modulation of the fluorescence signal with respect to the exciting light. The modern study of fluorescence properties started with time domain fluorometry and has evolved into methods using frequency domain fluorometry. In the frequency domain, the frequency axis is examined one point at a time, while in the  
10 time domain, the full decay is collected at once; however, the collection of information in the time domain takes from several minutes to several hours depending upon the excitation source, while in the frequency domain, the data collection at a single frequency takes only a few seconds.  
15 Therefore, it is possible in the frequency domain to acquire an equivalent amount of information in a similar amount of time. Indeed, a great advantage in the frequency domain can be achieved if all frequencies can be collected at the same time.

20 The maximum time resolution of sequential multifrequency phase fluorometers is about 1 or 2 picoseconds, which compares favorably with time correlated single photon counting instruments. The decomposition of the decay curve using a sum of exponentials, may also be obtained  
25 from a multifrequency measurement applying a non-linear least

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squares routine. The analysis of a double and triple  
exponential decay may be performed on dedicated  
1 micro-computers.

Resolution of emission anisotropy decay is obtained  
by a measurement of the differential phase and modulation ratio  
of the horizontally and vertically polarized emission  
5 components, arising from vertically polarized excitation.  
This technique, originally developed for single modulation  
frequency operation, has become extremely powerful when  
coupled with a multifrequency phase fluorometer. Fast  
rotational correlation times on the order of 10 picoseconds  
10 and longer can be measured. Resolution of anisotropic  
rotational motions can also be obtained from a multifrequency  
data set using a non-linear least squares analysis.  
Restricted rotational motions can also be analyzed. The  
ability to perform direct differential measurement, such as  
15 the phase delay between the perpendicular and the parallel  
polarized components of the emissions, is a unique intrinsic  
characteristic of phase fluorometry and results in an  
improved time resolution.

Phase fluorometry has the intrinsic capability to  
20 perform phase sensitive detection, which provides a simple  
and powerful method to separate spectral components in a  
mixture of fluorophores. This separation is based on the  
principle that each emitting species in the mixture has a  
characteristic phase delay. The spectra of the overlapping  
25 components can be obtained with a single scan using our new

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approach of phase and modulation resolved spectra. This  
simple approach requires no fitting of the data. The  
1 resolution is instead obtained directly from the values of  
the phase and modulation.

The prior art shows a number of examples of systems  
utilizing frequency domain fluorometry techniques. The 1984  
5 article "The Measurement and Analysis of Heterogeneous  
Emissions by Multi-frequency Phase and Modulation  
Fluorometry" by Jameson, Gratton, and Hall, Applied  
Spectroscopy Reviews, 20(1), pages 55-106 (1984) discloses  
10 two methods of multi-frequency phase and modulation  
fluorometry as well as a commercially available fluorometer.  
In addition, the article discloses a fluorometer the authors  
developed for research purposes. The commercially developed  
fluorometer, developed by SLM AMINCO, utilizes a xenon arc  
15 lamp to provide an excitation signal to generate the  
fluorescence emissions. The light supplied by the arc lamp  
is intensity modulated before impinging upon a sample to be  
studied. The light emitted by the (study) sample is detected  
by a photomultiplier, the last dynode of which is modulated  
20 at a frequency equal to the light modulation frequency plus a  
small additional frequency. This procedure is a  
cross-correlation technique, wherein the phase and modulation  
information of the emitted signal is transposed to a much  
lower frequency range where it can be interrogated. The  
phase delay and demodulation of the emitted signal relative  
25 to the scattered light is then calculated. The research

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fluorometer described in the article is a variable frequency cross-correlation phase fluorometer which utilizes an argon  
1 ion laser to provide an excitation beam to excite the  
fluorescence action and to provide a reference signal. The  
light supplied by the laser is sinusoidally modulated, and  
split into two beams, one signal is used to excite the study  
5 sample and the second signal is used as the reference signal.  
The reference signal and the signal emitted by the study  
sample are then passed through two photomultipliers wherein  
the cross-correlation processing described above is done.  
The outputs from both photomultipliers are then passed  
10 through identical sections of analog circuitry wherein the  
data is sequentially processed and displayed.

The 1986 article "A Multi-Frequency Phase  
Fluorometer using the Harmonic Content of a Mode Locked  
Laser" by Alcala and Gratton, Analytical Instrumentation,  
15 14(3 and 4), pages 225-250 (1985) discloses a  
cross-correlation phase and modulation fluorometer which  
utilizes the harmonic content of a high repetition rate, mode  
locked laser. In the frequency domain a pulsed source  
provides a large series of equally spaced harmonic  
20 frequencies. The pulses from the laser are amplitude  
modulated and frequency doubled. The signal is then split  
into a reference beam and an excitation beam. The reference  
beam is directed to a first photomultiplier and the  
excitation beam is directed to a study sample and then the  
25 emission from the sample is detected by a second

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photomultiplier. The photomultipliers provide cross-correlated mixing which in addition to frequency translation also allows transfer of the phase and modulation information desired at the individual harmonic frequencies. The outputs from the photomultipliers are then passed through various forms of analog filtering circuits and amplifiers wherein the necessary phase and modulated data is sequentially derived from the outputs of the photomultipliers.

Frequency domain fluorometry in certain instances has the advantage of the rapid determination of single or double exponential fluorescence lifetimes which can be obtained by measurements at only one or two frequencies. This is not possible for systems where complex fluorescence decays must be resolved. In order to handle complex decays, a large number of modulation frequencies is needed to obtain the full decay information. The above disclosed fluorometers provide this capability only to a limited extent.

The above referenced articles disclose fluorometers that use frequency domain techniques as opposed to time domain techniques. Frequency domain fluorometers have the advantage of high accuracy and rapid determination of fluorescence lifetimes. However, the above referenced fluorometers utilize analog signal processing techniques after data collection. Unwanted effects on the signals of interest are caused by the bandwidth and non-linearity of the analog filters used in the above referenced fluorometers. In the analog electronics of most commercial frequency domain

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fluorometers, six pole active filters are utilized to perform the necessary filtering functions. These filters are hard to  
1 tune to the appropriate frequency, they suffer from thermal and drifting problems and have undesirable phase shift. The accuracy of lifetime measurement is limited by the analog  
signal processing portion of the fluorometers.

5 SUMMARY OF THE INVENTION

The present invention is an array detector for detecting the modulated light emissions of an excited sample, said array detector comprising a semiconductor array for  
10 receiving and recording the level of modulated light emissions received from said sample; an image intensifier positioned between said sample and said semiconductor array, said image intensifier modulating the gain of the modulated  
light emissions received from said sample and translating  
15 said emissions from a first frequency range to a second frequency range; means for selectively biasing said image intensifier to effectively pass to said array detector  
emissions having frequencies of  $f_1$  and  $f_c$ ; and means for  
20 reading at a frequency greater than  $f_c$ , the recorded levels of modulated light emissions of said array detector.

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BRIEF DESCRIPTION OF THE DRAWINGS

1 Figure 1 is a diagrammatic illustration of one embodiment of our invention using photomultiplier tubes for cross-correlation mixing.

5 Figure 2 is a diagrammatic illustration of a second embodiment of our invention using an improved array detector for optical cross-correlation mixing.

Figure 3 is a schematic illustration of a current to voltage converter and amplifier used to match the output of the PMT tubes to a standard analog to digital converter.

10 Figure 4 is a diagrammatic illustration of the array detector used in one embodiment of the invention.

Figure 5 is a schematic illustration of the circuit used to insert the pulse and correlation frequency into the array detector illustrated in Figure 4.

15 Figure 6 is a graph illustrating the preferred biasing voltage between the image intensifier and the array detector.

Figure 7 is a conceptual illustration of the direct memory access portion of the invention.

20 Figure 8 is a simplified flow chart of the data acquisition and data processing programs used in the present invention.

Figure 9 is a graph illustrating the phase and modulation value of P-terphenyl obtained with the present invention.

25 Figure 10a is a graph illustrating the filter response of the digital averaging filter using 10 seconds of integration.

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Figure 10b is a graph illustrating the filter response of the fast fourier transform using only the  
1 fundamental frequency.

Figure 10c is a graphic illustrating the response of the combined averaging filter and fast fourier transform calculated for the fundamental frequency.

5 DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention relates to improvements in the field of frequency domain phase fluorometry. In one embodiment, a pulsed light source having a predetermined frequency and multiple harmonics is used to simultaneously  
10 excite a sample at a fundamental and a plurality of harmonic frequencies. Improved digital acquisition and cross-correlation techniques enable the collection of the phase and modulation information at each of the frequencies onto a single wave form. The wave form is digitally filtered to  
15 remove non-harmonic and non-synchronous frequencies, and a fast fourier transform is performed on the filtered waveform.

The result is the simultaneous derivation of the phase and modulation values of the sample response at a plurality of frequencies from a single excitation.

20 In a second embodiment, the first embodiment is used with an array detector capable of collecting discrete values of the phase and modulation response at a plurality of x-y locations, and at a plurality of various wave length or color emissions to assist in resolving and imaging multiple emissions  
25 from a single excitation.

The array detector provides an improvement over known array devices in as much as it enables measurements of the luminescence decay time in the pico-second to nano-second range over the entire spectral emission band using correlated  
30 gating techniques. The gating reduces the duty cycle of the

1 measurement, and extends the maximum resolution time to about  
20-30 pico-seconds with a duty cycle of about 50%.

### MULTI FREQUENCY PHASE FLUOROMETRY

5 The time decay of fluorescence is typically  
measured using one of two different approaches. The system  
response to transient (pulsed) excitation can be determined  
in the time domain by the popular technique of time  
correlated single photon counting. Alternatively, the  
fluorescence response can be measured in the frequency  
10 domain, by determining the phase delay and the relative  
modulation of the fluorescence signal with respect to the  
exciting light. The time domain and frequency domain  
approaches provide equivalent information and are related to  
each other by the fourier transform.

15 In the frequency domain the time variation of the  
excitation light intensity is described by

$$E(t) = E_0(1 + M_e \sin(\omega t)) \quad (1)$$

where  $E_0$  and  $M_e$  are the average value of the  
intensity and the modulation of the excitation respectively.

20 The overall fluorescence response of the system to sinusoidal  
excitation can be written in the form

$$F(t) = F_0[(1 + M_f \sin(\omega t - \phi))] \quad (2)$$

Where  $F_0$  and  $M_f$  are the average value of the  
intensity and the modulation of the fluorescence,  
25 respectively. For linear systems the emitted fluorescence  
has the same modulation frequency but is demodulated and  
phase-shifted with respect to the exciting light. The phase  
delay and modulation ratio between the excitation and the  
emission constitute the two independent measurable quantities  
30 in phase fluorometry. The following equations relate these  
parameters to the case of the pulse response,  $I_F(t)$ , to  
excitation by a delta function at excitation frequency,  $\omega$ ,

$$\tan \phi = \frac{S}{G} \quad (3)$$

$$M = M_e = N^{-1}(S^2 + G^2)^{1/2} \quad (4)$$

where

$$S = \int_0^{\infty} I_F(t) \sin \omega t \, dt \quad (5)$$

$$G = \int_0^{\infty} I_F(t) \cos \omega t \, dt \quad (6)$$

$$N = \int_0^{\infty} I_F(t) \, dt.$$

Knowledge of  $\phi$  and  $M$  is equivalent to knowledge of the functions  $S$  and  $G$  which correspond to the sine and cosine fourier transforms of the ideal pulse response  $I_F(t)$ .

Consequently the measurement of phase and modulation as a function of the frequency is equivalent to determining the time evolution of the emitting system to delta pulse excitation. In phase-modulation fluorometry, however, deconvolution for the finite width of the excitation pulse and the time response of the detection system is unnecessary since the ideal pulse response is obtained.

Multiple frequency excitation has traditionally been accomplished by using a synchrotron or pulsed laser output at a plurality of frequencies. It is also known that pulsed light sources contains multiple harmonics, and that in the frequency domain all of the photons in the light source contribute to the measurement of each harmonic frequency. The average signal measured at the  $i$ .th harmonic for very narrow pulses has practically the same intensity as the complete fluorescent signal.

Since the use of the preselected fundamental and harmonic frequencies obviates the need for sequentially collecting separate measurements at each frequency, and the attendant needs to tune and acquire "dark wave" reference

1 signals at each frequency, its use is preferred in the  
 practice of this invention, except where the measurement of  
 fluorescent lifetime or rotational rate requires the use of a  
 frequency available only from a modulated source.

# 5 The Principle of Cross-Correlation Parallel Phase Fluorometry

Cross-correlation in a phase fluorometer was first  
 described by Spencer and Weber in an article entitled  
 "Measurements of Sub-nano-second Fluorescence Lifetime with a  
 Cross-Correlation Phase Fluorometer", Ann. New York Acad.  
 Sci. (1969) p361. In the present invention, the operating  
 10 principle is the same, but it is extended to cover the  
 harmonics in the cross-correlation signal. When a  
 fluorophore is excited by a pulsed light source, the  
 fluorescence has the same frequencies as the excitation, but  
 15 each harmonic frequency is demodulated and phase shifted  
 differently with respect to the exciting light. The  
 modulation ratio,  $M$ , and the phase shift,  $\phi$ , are related to  
 the fluorescence lifetime,  $\tau$ , by

$$20 \quad \tan \phi = \omega \tau \quad (7)$$

$$M = \frac{M_f}{M_e} = \frac{1}{\sqrt{1+(\omega\tau)^2}}.$$

25 where  $M_f$  and  $M_e$  are the modulation of the fluorescence and  
 the excitation respectively. The frequency content of the  
 fluorescence can be written as

$$F(t) = F_0 \left[ 1 + \sum_{n=1}^N M_{fn} \cos (n\omega t + \phi_n) \right], (8)$$

30 where  $F_0$  is the average fluorescence. The cross-correlation  
 technique mixes the fluorescence signal with a

1 cross-correlation signal,  $C(t)$ , which is at a slightly  
different base frequency,  $w_c$  :

$$5 \quad C(t) = C_0 \left[ 1 + \sum_{k=1}^K M_{ck} \cos(k\omega_c t + \varphi_k) \right], \quad (9)$$

the resulting signal is the product of  $V(t) = F(t) \cdot C(t)$ .

$$10 \quad V(t) = F_0 C_0 \left[ \left( 1 + \sum_{n=1}^N M_{fn} \cos(n\omega t + \varphi_n) + \sum_{k=1}^K M_{ck} \cos(k\omega_c t + \varphi_k) \right) \right. \\ \left. + \sum_{n=1}^N M_{fn} \cos(n\omega t + \varphi_n) + \sum_{k=1}^K M_{ck} \cos(k\omega_c t + \varphi_k) \right]. \quad (10)$$

15 The last term can be rewritten using trigonometric  
relationships as the sum and difference of the two  
frequencies. If we look at only the lowest frequency region,  
with  $i=j$ , the only term remaining is:

$$20 \quad \sum_{n=1}^K \frac{M_f M_c}{2} \cos(n\Delta\omega t + \Delta\varphi). \quad (11)$$

where  $\Delta\omega = \omega_c - \omega$ ,  $\Delta\varphi = \varphi_i - \varphi_i$ . This series ends at  $n=K$  since we  
have assumed  $K < N$ , i.e. the cross-correlation signal has less  
harmonic content than the fluorescence signal. This  
expression contains all of the phase and modulation  
information of the original fluorescence signal at all the  
25 harmonic frequencies, now as harmonics of  $w_c$ , but if  $w_c$  is  
very close to  $w$ , then this information is at much lower  
frequencies that are easier to isolate and sample with our  
digital electronics. In the embodiment illustrated in Figure  
1  $\Delta\omega$  was set to 40 Hz. In the embodiment illustrated in  
30 Figure 2,  $\Delta f = w/2T$  is set to 15 or 7.5 Hz.



1 For parallel phase fluorometry, a high harmonic  
content in both the light modulation and in the  
cross-correlation signal is required. High repetition pulsed  
sources, such as mode-locked lasers and synchrotron  
5 radiation, intrinsically contain a high harmonic content.  
Traditionally, the cross-correlation product is obtained by  
applying an appropriate voltage to one of the dynodes of the  
photomultiplier tube. This internal mixing is quite  
powerful, since the PMT itself is a very good mixer. The PMT  
10 dynode chain produces good amplification with very low noise,  
and it does not require any extra components. In the  
embodiment illustrated in Figure 1, the mixing occurs in the  
photomultiplier tube. In the embodiment illustrated in  
Figure 2, the mixing occurs in the light signal emitted by  
15 the sample by a gating technique, prior to the sampling by  
the diode array. A more complete explanation of the cross  
correlation accompanies the detailed description of each  
embodiment.

#### PARALLEL PHASE FLUOROMETER

20 The parallel phase fluorometer illustrated in  
Figure 1 has the intrinsic capability to separate out all of  
the harmonic information contained in the cross-correlated  
signal. This capability can be exploited by using a light  
source that has high harmonic content, such as a pulsed laser  
25 system, or by pulsing the Pockel's cell modulator used in  
most phase fluorometers and cross-correlating with a waveform  
that contains harmonics. A mode-locked laser system is also  
known to have a frequency content of several gigahertz, and  
pulsing other light modulation systems can increase their  
30 frequency content. Laser diodes and high speed light  
emitting diodes intended for use with fiber optics may also

1 be used to generate a pulsed wave form with a high harmonic content.

By using a light source with an intrinsic high harmonic value, the total data acquisition time can be greatly decreased by simultaneously acquiring many frequencies. In the embodiment illustrated in Figure 1, laser 11 is a mode locked Nd-YAG laser which synchronously pumps a cavity dumped dye laser 12 (Antares model, 765-700 Coherent). The pulse train output is frequency doubled to UV light by using a frequency doubler 13, Coherent model 7049.

This laser system can cover the wavelength range from 265 nm to 850 nm by changing the laser dyes and the doubler crystal. The repetition frequency of the laser is normally set from 1 to 2.00 MHz. All harmonics of the basic frequency can be used up to about 1000MHz, (a limit imposed by present synthesizers and radiofrequency amplifiers). If a lower modulation frequency is required, the cavity dumper can be set to any submultiple of 1 MHz, up to a single pulse operation.

The polarization of the dye laser light is vertical relative to the laboratory axis while the UV output from the doubler 13 is horizontally polarized. The plane of polarization of the UV beam is rotated to 35 degrees from the vertical (the ideal polarization angle for lifetime measurements) using an arrangement of two mirrors. The mirrors 14, 15 which not only change the polarization angle of the exciting light but also steer the beam into the optical module 16, and have a metallic coating (Melles Griot coating 028); since a dielectric coating would give rise to a wavelength and polarization dependent reflection. The emission of the sample may be polarized by polarizer 17 for measurement and study of anisotropic decay. The optical

1 module 16 also includes a quartz beam splitter 18, filter  
holders 19, 20 and a sample receiving holder 21.

Hamamatsu R928 photomultiplier tubes 22,23 are  
selected because of their wide-range wavelength sensitivity,  
5 high gain, low price and relatively small color effect.

The modulation of the PMT tubes 22,23 is obtained  
by application of an alternating voltage to the second dynode  
D2 through a power splitter 24, as illustrated at 25,26. The  
characteristic curve of the PMT has a sharp rise, then the  
10 current reaches a maximum and decays again as the absolute  
voltage of the dynode increases. In order to modulate the  
gain of the PMT an RF voltage of about 40V peak to peak is  
needed corresponding to an average power of 4W on a 50 ohm  
terminator. The RF voltage is provided by an RF amplifier 27  
15 (ENI Model 603L.)

Instead of attempting to acquire the entire  
frequency range in one measurement, we acquire the range from  
1 MHz to 500 MHz in three steps. The laser is mode locked  
at  $F_1$  by frequency synthesizer 28, while the PMT's are pulsed  
20 at  $F_1 + 40\text{Hz}$ , 40 Hz is the cross correlation frequency by  
frequency synthesizer 29. The synthesizers 28,29 are  
maintained in a phase locked loop figuratively illustrated at  
30. In normal operation, the synthesizer 28, is set at a  
frequency of 1 MHz with a pulse width of 100 ns. Synthesizer  
25 29 is set at  $1\text{ MHz} + 40\text{Hz}$ . The pulse width is 100 ns.

The duty cycle becomes 1/10 with a reduction of  
only a factor of 5 with respect to the standard single  
frequency mixing (duty cycle 1/2). Using this pulsed  
cross-correlation signal, about nine different frequencies  
30 can be easily collected in the range from 1 to 9 MHz. Then,  
the base frequency of the synthesizers are set at 10 MHz, and  
10 MHz + 40Hz with a pulse width of 10 ns and the duty cycle

1 is still 1/10. Again, frequencies are collected in the range  
from 10 to 90 MHz. Finally, the base frequency of the  
synthesizers are set at 100 MHz and 100 MHz + 40Hz and the  
pulse width to about 2 ns and frequencies are collected from  
5 100 MHz up to about 250 MHz. This frequency limit is imposed  
by the PMT detectors and by the fluorescence characteristic  
of the emitting substance. The reduction in acquisition time  
with respect to the prior art sequential multi-frequency mode  
is about a factor of ten, since ten frequencies are collected  
10 simultaneously.

The output of PMT tubes 22,23 carry the phase and  
modulation information imparted by the sample on a  
correlation frequency of 40Hz. The cross-correlation current  
signal on signal lines 31, 32 is first converted to a voltage  
15 signal, and then amplified by amplifiers 314, as more fully  
explained with respect to Figure 3. The amplified signals  
are then digitized at 700, as hereinafter explained.

#### THE DIGITAL ACQUISITION SYSTEM

In the digital acquisition system of our invention,  
20 most of the analog electronics have been eliminated. The  
only analog elements used are the current-to-voltage  
converters 300 needed to transform the output of the  
photomultiplier tubes to a voltage and the amplifiers 314, to  
boost the signal level. The current-to-voltage converter and  
25 amplifier are built directly into the empty slot of a  
commercially available data acquisition board. In one  
embodiment of the invention, a MicroWay A2D-160 board was  
used because of its speed, its two collection channels, and  
its use of the computer's direct memory access (DMA)  
30 capabilities. This board fits into a slot of any  
IBM-PC-compatible computer. Direct memory access relieves  
the central processing unit (CPU) from processing data during

1 the acquisition period, so that data collection and storage  
occur in the background. Therefore, the CPU is used only for  
the digital filtering processes and determination of the  
phase and modulation values of previously acquired waveforms.  
5 The CPU is free most of the time to run normal "housekeeping"  
tasks, such as displaying information on the status of the  
instrument. The A2D-160 board has a 12-bit analog-to-digital  
converter with a maximum sampling rate in single channel mode  
of 160 KHz. In our experience, 12-bits were always  
10 sufficient to obtain good accuracy. The actual resolution is  
improved due to the noise level of our signal. We have  
estimated that in our experimental condition we have about  
15- to 16-bit effective resolution. With respect to the  
sampling rate, we are well below the board's limits. For the  
15 measurements reported here, we have used a sampling rate of  
2.56 KHz.

Referring now to Figure 3 the analog circuitry is  
illustrated in schematic form. The output signals of the  
photomultiplier tubes enter the current-to-voltage converter  
20 300 through a 50 ohm resistor 302 and a 1 megaohm resistor  
304 to ground combination. The signal continues through this  
combination to an active low pass filter comprised of an  
operational amplifier 306 with a negative feedback path  
comprised of a parallel combination of a 1 megaohm resistor  
25 308 and a 1000 picofarad capacitor 310, the effect of which  
is to attenuate higher frequency signals. The operational  
amplifier is powered by a positive 12 volt signal 301 and a  
negative 12 volt signal 303. A variable 10 kilo-ohm resistor  
312 is used to adjust the zero offset of the operational  
30 amplifier 306. The operational amplifier 306 used is an  
AD515 manufactured by Analog Devices, Inc.

1           The output of the current-to-voltage converter 300  
is then directed to a variable gain amplifier 314. The  
variable gain amplifier 314 is capable of amplifying the  
output of the current-to-voltage converter 300 by a magnitude  
5 of 1, 10, 100 or 1000 times. The variable gain amplifier 314  
is powered by a positive 12 volt signal 305 and a negative 12  
volt signal 307. Adjustments to the variable gain amplifier  
314 are made through a pair of 10 kilo-ohm resistors 316 and  
318 which are connected to the positive and negative 12 volt  
10 signals and to ground through a pair of 1 microfarad  
capacitors 320 and 322. The value of the gain on the  
variable gain amplifier 314 is determined by a signal  
generated by a bank of relays 324.

          The bank of relays is comprised of three  
15 independent relays 326, 328 and 330. The relays 326, 328 and  
330 are controlled by digital logic circuitry comprised of  
three integrated circuits 332, 334 and 336. Each relay 326,  
328 and 330 is comprised of a set of diodes 321, 323 and 325  
a set of coils 327, 329 and 331 and a set of switches 333,  
20 335 and 337. One side of each diode 321, 323 or 325 is  
connected to a positive 5 volt source 339 while the other  
side is connected to a digital integrated circuit 334 via  
lines 341, 343 and 345. When the value of any of the three  
outputs of integrated circuit 334 are logic 0, which  
25 corresponds to 0 volts, then that particular diode 326, 328  
or 330 conducts current and magnetizes the particular coil  
327, 329, 331 and thereby closes switches 333, 335 or 337.

          The digital circuitry comprised of three integrated  
circuits 332, 334 and 336 control the gain on amplifier 314  
30 by controlling the relays 326, 328 and 330. Integrated  
circuit 332 is a series of four NAND gates 338, 340, 342 and  
344 which receive an I/O SELECT and I/O WRITE signal from the

1 host computer. These two signals are used to latch the  
integrated circuit 336. Integrated circuit 336 receives  
three input signals D0, D1 and D2 from the host computer.  
These three signals are latched and stored in the integrated  
5 circuit 336 and are output to three inverters 346, 348 and  
350 which are realized on one integrated circuit 334. By  
varying the possible combinations of D0, D1 and D2, the gain  
on the variable gain amplifier 314 can be altered. Table 1  
10 given below contains the combinations of D0, D1 and D2 and  
the gains they cause to be made.

GAIN TABLE

	<u>D2</u>	<u>D1</u>	<u>D0</u>	<u>GAIN</u>
	1	1	1	1
	1	1	0	10
15	1	0	1	100
	0	1	1	1000

In the digital acquisition system of the present  
invention, the host or controlling program is an adaptation  
20 of the standard acquisition software used in our laboratory  
and is available through Globals Unlimited, Department of  
Physics, UIUC. The program starts by initializing the  
hardware and setting up data files. First, the  
analog-to-digital board is disabled and the on-board timer is  
25 programmed. The A2D-160 card has a 4 MHz clock which is used  
by an AM9513 counter chip from Advanced Micro Devices. A  
"master reset" is issued to the AM9513, this resets and stops  
all counters; counter one is then loaded. This counter  
divides the 4 MHz clock to provide the appropriate sampling  
30 rate for the cross-correlated signal, which we have chosen to  
be at 40 Hz. Next, the DMA channel 1 of the IBM PC is

1 masked, and programmed to transfer 2560 data points from the  
analog-to-digital card to a storage vector in the main  
computer memory. The 2560 data points represent 1280 data  
points per channel, which correspond to 32 data points per  
5 period for 40 periods. The 32-data points-per-period was  
chosen because the highest harmonic that the fast fourier  
transform (FFT) algorithm, used by the filtering routine, can  
resolve is equal to half of the number of data points. The  
possibility to analyze up to the sixteenth harmonic was felt  
10 to be high enough for our application. This is not a  
limitation, because the number of data points per period can  
be increased with only a linear penalty of computation speed.  
The 40-period integration range was chosen because at the  
cross-correlation frequency of 40 Hz used in our instrument,  
15 data are collected in one second, and also for the efficiency  
of the filtering, which will be discussed later. Channel 3  
on the PC interrupt controller is masked, and a new interrupt  
vector, pointing to a display and save routine, is loaded.  
When the timer, the DMA, and the interrupt controller have  
20 been programmed, the DMA and interrupt controller are  
unmasked, and the timer is started. The timer is  
free-running, so data are collected asynchronously.

The data collection proceeds, simultaneously  
converting both the excitation and emission channels by using  
the two independent sample-and-hold circuits, and is sampled  
25 by the 12-bit analog-to-digital converter with a full scale  
range of -5 to +5 volts. As illustrated in Figure 7, at the  
end of the conversion process the DMA 702 is addressed. The  
DMA then transfers the output of the analog-to-digital  
30 converter into the main memory of the computer as illustrated  
at 704; then the other sample-and-hold circuit is read,  
converted, and stored. The whole cycle is repeated until the



1 2560 data points are collected. Once the data has been  
stored, the DMA generates an end-of-process which triggers  
the interrupt routine indicated at 706. The interrupt  
routine 706 folds the 40 periods that arise from the one  
5 second integration into one, and then reduces the 32 data  
points into four bins, representing four phases of a period  
at the lowest harmonic frequency. The DC, AC, modulation,  
and phase of the waveform can be rapidly calculated from the  
values of the four bins. Those values are used only to give  
10 basic information "on the fly" for the data being collected.  
This information is displayed at the top line of the computer  
screen, and is updated every second. This information is  
useful to the user for continuous monitoring of the measuring  
conditions of the instrument. The interrupt routine  
15 reprograms the DMA and the interrupt controller and restarts  
the counter. The cycle starts again and is continuously  
repeated.

At the beginning of a measurement, the program sets  
the basic frequency of the synthesizer and asks for the  
20 reference lifetime value. A dark waveform is then digitized  
by repeating the interrupt cycle ten times. After the data  
have been collected, the averaged and folded waveform is  
analyzed by a FFT routine which provides additional  
filtering. The real and imaginary parts of the FFT are  
25 sufficient to calculate the AC, DC phase and modulation of up  
to the sixteenth harmonic. These values are subtracted from  
the sample and reference waveforms to reduce in-phase pickup  
noise. After the dark waveform is measured, the sample is  
illuminated and the fluorescence signal is acquired. The AC,  
30 DC, phase and modulation values are determined at the same  
moment. The reference compound (lifetime = ref) is then  
illuminated, and its AC, DC, phase and modulation values are

1 calculated. When both the sample and reference have been  
collected, absolute phase and modulation values are  
calculated using the following expressions.

5 
$$M_{\text{corr}} = \frac{M_{\text{sam}}}{M_{\text{ref}}} \frac{1}{\sqrt{1 + \omega^2 \tau_{\text{ref}}^2}}$$

and

$$\phi_{\text{corr}} = \tan^{-1}(\omega \tau_{\text{ref}}) + (\phi_{\text{sam}} - \phi_{\text{ref}}).$$

10 The sample is again illuminated, and its modulation and phase  
values are determined. Absolute phase and modulation values  
are then calculated using the new values of the sample  
modulation and phase and old reference numbers. The  
15 corrected modulation and phase numbers are averaged together,  
and the standard deviation is calculated. The reference  
sample is then illuminated and the cycle is repeated until  
the variance is below 0.2 degree and 0.004 for the phase and  
modulation, respectively.

20 This entire process is automatically controlled by  
the on-line computer using the Globals Unlimited software  
described above, as driven by the executive level software  
described in Figure 8, and attached hereto as Appendix A.

Referring to Figure 8, the entire procedure is  
25 shown in flow chart format. The data collection as described  
above is shown by block 802 of the flow chart. After the  
collection of data, the software checks to see if the EOP  
interrupt has been received thus indicating that the data is  
ready. This is represented by block 804 and corresponds to  
30 line 11 through line 40 on page 3 of the computer program  
listing as set forth in Appendix A. If the interrupt has  
been detected, the CPU transfers control to the interrupt

1 routine indicated by the dotted box 803. The first box 806  
represents the portion of the interrupt software that is  
responsible for folding the acquired waveform into one period  
of time, the reasons for this are explained earlier. The  
5 software that corresponds to box 806 is given in line 45 on  
page 3 of the computer program listing through line 2 on page  
4 of the computer program listing. Block 808 represents the  
portion of software responsible for calculating the discrete  
fourier transform of the collected waveform. The software  
10 corresponding to box 808 is given in line 60 on page 10 of  
the computer program listing through line 60 on page 11 of  
the computer program listing. Block 810 represents the  
portion of the software that is used to calculate the DC, AC,  
modulation and phase of the wave form. The software  
15 corresponding to box 810 is given in line 65 on page 11 of  
the computer program listing through line 36 on page 12 of  
the computer program listing. Block 812 is a routine that  
displays the information calculated in block 810. The  
software corresponding to box 812 is given in line 20 on page  
20 4 of the computer program listing through line 20 on page 5  
of the computer program listing. The information is  
displayed at the top line of the computer screen, and is  
updated every second. Block 814 represents the software used  
to reprogram the DMA 702 shown in Figure 7, the interrupt  
25 controls and it also restarts the internal counter. The  
software corresponding to box 814 is given in line 25 on page  
5 of the computer program listing through line 20 on page 10  
of the computer program listing. Upon completion of the  
routine described in box 814, the software is now returned to  
30 the main software routine. Block 816 copies the information  
calculated by block 810 into new variables for further  
manipulation.

1           When both the sample and reference waveforms have  
been collected as described above, absolute phase and  
modulation values are calculated, which is represented by  
block 818. The corrected modulation and phase numbers are  
5 averaged together to form average values as is shown in block  
820. The processing corresponding to boxes 816, 818 and 820  
is done in a software loop given in line 60 on page 12 of the  
computer program listing through line 59 on page 13 of the  
computer program listing. After averaging the values  
10 together, the standard deviation is calculated and checked to  
see if it is in the specified range as shown in decision box  
822. If standard deviation is not acceptable, the process of  
analyzing the data is repeated. If standard deviation is  
within tolerance, the software returns to its starting point,  
15 illustrated by block 824. The calculation of the standard  
deviation and the software corresponding to decision box 822  
is given in line 61 on page 13 of the computer program  
listing through line 15 on page 14 of the computer program  
listing.

20           The digital acquisition system of the present  
invention excels at filtering. This operation must reject  
random and harmonic noise. Simulations show that if the  
second harmonic has an amplitude of 0.05 of the fundamental  
after the filtering, and is incorrectly associated with the  
25 first harmonic, the resulting phase measurement can be off by  
as much as five degrees. This is a very large error, and  
therefore the harmonics must be reduced to less than one part  
in 200 for the effect to be less than 0.2 degrees. In the  
standard analog electronics of most commercial frequency  
30 domain fluorometers, six pole active filters are used to  
perform the appropriate filtering. These filters are hard to  
tune, suffer thermal drifting problems, and have

1 amplitude-dependent phase shifts, which become a problem if  
the sample and reference compounds do not emit nearly equal  
amounts of light. If this is the case, then the signal out  
of the PMT will have different amplitudes for the sample and  
5 reference cuvettes and the resulting phase-shifts from the  
filters will introduce a systematic phase deviation. The  
digital acquisition system of the present invention uses a  
sequence of two digital filters that do not suffer from these  
problems.

10           The first digital filter is the averaging filter.  
Since data are collected by acquiring 40 periods in a  
continuous stream and folding into one period, any frequency  
that is not a harmonic of the fundamental will destructively  
interfere with itself. Also, all signals which are not  
15 synchronous with the fundamental will average out. For  
example, if the fundamental is at 40 Hz and a 20 Hz signal is  
added, then in one 40 Hz waveform there is one-half of the 20  
Hz waveform and the next 40 Hz waveform will contain the  
opposite half of the 20 Hz waveform. When the two waveforms  
20 are folded and added, the 20 Hz signal will cancel out  
exactly and the 40 Hz signal will remain. The filtering  
action of this filter depends on the number of waveforms  
collected and folded. The experimental filter response of  
our 40 waveform-averaging filter is shown in figure 10a. The  
25 points for this figure were obtained by applying a sinusoidal  
signal out of a HP3525 synthesizer directly to the A2D-160  
board and then varying the frequency over the range specified  
in the Figure.

30           An inherent property and, as we show later, an  
advantage of the averaging filter is that it lets the  
harmonics pass through. To separate the fundamental and the  
harmonic information, the averaging filter's output is

1 processed by a FFT routine. The FFT routine also acts as a  
filter, because it resolves the input waveform to a DC value,  
the fundamental frequency, and its harmonics. Therefore, any  
of the harmonic frequencies can be rejected by simply  
5 ignoring its output from the FFT. The experimental filter  
response of the FFT, retaining the fundamental frequency  
only, is shown in Figure 10b. The same signal as in Figure  
10a was used to obtain the experimental points in Figure 10b.  
The FFT also provides the values needed to calculate the  
10 phase and modulation of the acquired waveform. The two  
filters, the averaging and the FFT, are in series and the  
final result is the product of the two filters. The total  
filter response, for the fundamental, is shown in Figure 10c.  
As can be seen, the harmonics are rejected by more than a  
15 factor of 2,000. This is an improvement over the analog  
electronics of about a factor of ten.

To illustrate the advantages of the digital filter  
over the analog electronics, we used both methods to perform  
a series of measurements of phase and modulation values as a  
function of the amplitude of an input signal. The input  
20 signal was composed of a basic frequency of 40 Hz plus a  
uniform noise band limited to 1000 Hz of 100 mV amplitude.  
The signal at 40 Hz was varied in amplitude while the noise  
level was left constant. The phase of the reference with  
25 respect to the sample channel was 180° to avoid the  
indeterminate region of the 0° to 360° for the analog  
acquisition mode, which would introduce additional phase  
noise. Above 1 V signal, both methods provided an adequate  
response: the average deviation and the standard deviation  
30 of the phase value were within 0.1°, a value commonly  
considered adequate for frequency domain fluorometry. When  
the signal-to-noise ratio became smaller, the performance of

1 the digital acquisition system was clearly superior to the  
analog electronics. The experimental conditions used in this  
test were typical of most of the measurements in frequency  
domain fluorometry where the signal-to-noise ratio is  
5 generally about ten.

#### Examples

A typical measurement from the fluorometer of the  
present invention is shown in Figure 9. The phase and  
modulation values for a solution of P-terphenyl in alcohol  
10 are shown together with the best fit for a single exponential  
decay. The excitation source is a mode-locked Nd-YAG laser  
which synchronously pumps a dye laser (Antares model,  
Coherent, Palo Alto, California). The output of the dye  
laser is cavity dumped and doubled to obtain ultraviolet (UV)  
15 light pulses. This pulse repetition rate is exactly 2.00  
MHz. The quality of the data acquired in parallel, using a  
10 second integration time for each of the three base  
frequency acquisition modes is better than the data obtained  
by the standard sequential mode using the analog electronic  
20 acquisition and 10 seconds integration time for each point.  
Note that with the parallel mode the entire decay was  
acquired in 60 seconds, as compared with 540 seconds  
effective integration time for the normal sequential mode.  
The actual acquisition time in the normal sequential mode was  
25 much larger (about 1000 seconds) due to the overhead time in  
manually setting the synthesizers to each new frequency and  
the need to acquire a dark current reading for every  
frequency.

The digital acquisition method described with  
30 respect to this invention allows for much better signal  
filtering than the analog electronics currently used in  
frequency domain fluorometers and also provides for the added

1 capability of parallel frequency acquisition. Another  
advantage of the digital electronics is the intrinsic  
capability to modify the base filter frequency by simply  
entering into the computer a different number for the  
5 acquisition period. Using this possibility, we have been  
able to determine the best cross-correlation frequency to be  
used on the basis of the phase noise characteristic of the  
synthesizer.

#### Parallel Phase Fluorometer with Array Detector

10 Figure 2 illustrates the parallel phase fluorometer  
of the present invention with a different detection and cross  
correlation means. As illustrated in Figure 2, the light  
source is a mode locked in Nd-YAG laser 50 which  
synchronously pumps a cavity dumped dyelaser 51 in a manner  
15 similar to that illustrated previously with respect to Figure  
1. The pulse train out is frequency doubled to UV-light by  
using a frequency doubler 52. Lasers 50, 51 are driven by  
mode lock driver 53 and cavity dump driver 54 which are in  
turn driven by a radio frequency amplifier 55. Frequency  
20 synthesizer 56 provides the driving frequency for the pulsed  
light source, while frequency synthesizer 57 provides the  
driving frequency for the cross correlation means. Frequency  
synthesizers 56 and 57 are phase locked with a phase lock  
loop with frequency synthesizer 56 generating a first  
25 predetermined fundamental frequency  $f_1$ , and frequency  
synthesizer 57 generating a second frequency, which includes  
 $f_1 + f_c$  wherein  $f_c$  is a correlation frequency. While  $f_1$  is  
selected to insure a high number of intrinsic harmonics,  $f_c$   
is selected primarily for compatibility with the array  
30 detector as will be hereinafter further discussed.

The polarization of the dyelaser light is vertical  
relative to the laboratory axis while the UV output from the



1 doubler 52 is horizontally polarized. The plane of  
polarization of the UV beam is rotated to 35 degrees from the  
vertical (the ideal polarization angle for lifetime  
measurements) using an arrangement of two mirrors, 58, 59,  
5 which not only change the polarization angle of the exciting  
light, but also steer the beam into the optical module 60.  
The mirrors 58, 59 have a metallic coating since a dielectric  
coating would give rise to wavelength and polarization  
dependent reflection. The pulsed light beam is split by beam  
10 splitter 61 into a reference beam 62 and a sample beam 63.  
The sample beam 63 impinges on a sample contained in sample  
holder 64 and the scattered light is passed through an  
aberration corrected polychromator 65 to the array detection  
system. The reference beam 62 is directed to a scattering  
15 surface 66, the output of which is measured by a reference  
detector 67 which may be a photomultiplier tube as was  
previously described with respect to Figure 1. The  
photomultiplier 67 mixes the output of RF amplifier 68 and  
the signal generated by reference beam 62 to derive a  
20 reference signal on signal line 69 which is essentially the  
correlation signal plus any system noise or deviation not  
related to the sample. The array detector will be more fully  
described with respect to Figure 4, it is comprised of three  
principle parts, image intensifier 70 a phosphorous layer 71  
25 and a semiconductor array detector 72. In the embodiment  
illustrated in Figure 2, the array detector was an Optical  
Multichannel Analyzer, Princeton Instruments, model  
IRY-512g/rb with an ISIT gatatable proximity focused  
micro-channel plate (MCP) image intensifier, that is  
30 optically coupled to a diode array.

The array detector 72 provides a sequential analog  
output on signal line 73 at a preselected frequency, varying  
from 30 to 120 sweeps per second. The image intensifier 70

1 is normally used to increase the gain of the array detector  
72. However, as used in the present invention, the biasing  
network for the image intensifier normally holds the  
photocathode potential at approximately 180 to 200 volts more  
5 negative than that of the potential of the microchannel  
electron intensifier 70. As driven by radio frequency  
amplifier 68, however, the cathode is driven to a gated mode  
wherein it is approximately 20 to 40 volts more positive than  
the image intensifier and effectively acts as a gate to  
10 prevent the light from the sample from reaching the array  
detector 72. The cathode in front of the image intensifier  
is gated closed at a rate  $f_1 + f_c$  determined by radio  
frequency amplifier 68 and frequency synthesizer 57. The  
sample is excited at frequency  $f_1$  by sample beam 63 and the  
15 emission spectrum from the sample is also varying at a  
frequency  $f_1$ , with certain phase and modulation relationship  
with respect to the excitation. The emission spectrum at  $f_1$ ,  
and the optical gating of the image intensifier at  $f_1 + f_c$   
creates two optical frequencies corresponding to the sum and  
20 difference of  $f_1$  and  $f_c$ . Since  $f_c$  is selected to be  
relatively low, on the order of 15 Hz, a signal at this  
frequency is received by the array detector 72. A sweep rate  
of 120 sweeps per second of the array detector results in a  
8X sampling of the 15 hertz correlation frequency imposed on  
the array detector 72 by the optical cross correlation of the  
25 image intensifier 70. Each complete cycle of the correlation  
frequency at 15 hertz carries the complete phase and  
modulation information imparted by the sample to the sample  
beam 63 by the emission characteristics of the sample.

30 The operation of the analog to digital converter,  
the data value acquisition programs, the averaging filter and  
the fast fourier transforms are essentially identical to that

1 described for Figure 1, with exception of the averaging or  
folding period. The differences relate to the differences in  
the filtering and averaging as necessary to accommodate the  
shift from 40 hertz to 15 hertz in the folding and averaging  
5 steps.

Figure 4 illustrates in a more figurative manner,  
the array detector illustrated at 70-72 in Figure 2. As  
illustrated in Figure 4, the device includes a quartz or  
optical fiber window 75, a photocathode 76 driven by input  
10 line lead 77, a microchannel plate electron intensifier 78  
which is nominally biased with in and out leads 79, 80. The  
array detector also includes phosphor layer 81 positioned  
between the image intensifier and the diode array 82. The  
phosphor layer and the diode array are coupled by means of an  
optical fiber coupler 83. The diode array 82 is a standard  
15 diode Reticon<sup>TM</sup> detector and array by Princeton Instruments .

The biasing network for the array detector  
illustrated in Figure 4 is illustrated in Figure 5 wherein a  
high voltage intensifier bias is imposed at 84. The -6.8KV  
20 is supplied by a power supply, not shown in Figure 2, to the  
cathode through a series of high resistances. The resistors,  
not shown, are for current limiting. The dc signal path from  
the cathode (not through any capacitors) goes right through  
to D1 and D2. The purpose of D1 and D2 is to accelerate the  
25 electrons to the grid. It is important to note that this  
device is never really turned off. This will be explained  
shortly. The RF signal from amplifier 68 is inserted at 85  
and the photocathode is biased as indicated at 77. The  
change produced by the incoming RF on the gain of the image  
30 intensifier through the acceleration voltage provides the  
modulation of the optical signal. The circuit illustrated in  
Figure 5 is an adaptation of the original circuit provided by

1 Princeton Instruments with the Optical Multichannel Analyzer.  
Zenor diode 86 is added to bias the photocathode 76 to the  
middle of the OMA gain curve as illustrated in Figure 6.  
Diode 86 is used to modify the voltage between the cathode  
5 and the MCP. The value of this diode is chosen in order to  
alter the gain by approximately a factor of 2. By reducing  
the gain by a factor of 2, the electrons are accelerated at a  
much lower rate in operation, the dynamic range of the OMA  
utilized is from approximately 40 indicated at A to 180  
10 indicated at A' in Figure 6. The use of a 90 volt zenor  
diode 86 provides a biasing voltage and an AC peak-to-peak  
voltage of approximately 60 volts as indicated by B-B' in  
Figure 6. As indicated previously, the photocathode  
potential on line 77 is normally set by the biasing circuit  
15 to be approximately 180 to 200 volts more negative than the  
potential of the microchannel plate 78. By biasing the  
photocathode with zener diode 86, the excursions of the radio  
frequency input signal at 85 raise the potential of the  
photocathode to approximately 20 to 40 volts more positive  
20 than that of the image intensifier 78, thereby effectively  
gating the image intensifier and preventing any of the  
emissions from sample in sample holder 64 from reaching  
either the phosphor layer 81 or the diode array 82.

25 All capacitors, except for the two coming in at the  
RF input are used for stability purposes. The average value  
of the cathode voltage should not change; therefore, the  
capacitors are used to stabilize this average value.

30 The remaining two capacitors, decoupling capacitors  
are to prevent the -6.8KV cathode voltage from leaking into  
the RF signal.

The use of the array detector, as illustrated in  
Figure 2, enables the separation of emission spectrum in at

least two different ways. First, the emissions may be separated by their spectral content since the OMA is connected  
1 by the polychromator 65, and the xy location of the emission can be tagged with the spectral response. Further, by combining the x,y coordinate information with the time resolved information, multidimensional information about a  
5 specific spectrum may be calculated. This information can subsequently be combined with the phase and modulation information derived by the processing means from the fast fourier transform to further assist in the separation of characteristic emissions in a mixed or multicomponent media.

10 Parallel array detectors (as was stated previously) are used for cross-correlation phase and modulation fluorometry, in conjunction with a modulated image intensifier; however, there are numerous other applications in which an array detector can be used. The modulation of the  
15 photo-electron current inside the intensifier is necessary to down convert or frequency translate the phase and modulation information, which is present in the fluorescence signal at high frequency typically in the mega hertz range, to a frequency typically in the hertz range, that can pass through  
20 the phosphor at the end of the intensifier and is below the Nyquist limit, which is the minimum sampling rate allowed to reconstruct a band limited waveform without error, set by the read rate of the detector. Typically, the phosphor has a decay time of 1 ms which limits the cross-correlation  
25 frequency to a value below 1 KHz. In normal photomultiplier based fluorometers, the cross-correlation frequency is at 40 Hz, so the phosphor is not a problem. The typical read rate per pixel on the phosphor is 33.33  $\mu$ s and thus for a 512 linear diode array, the Nyquist limit is 30 Hz, which is also  
30 acceptable. However, for a 500x300 CCD, the Nyquist limit is on the order of a second for a digitizer rate of about 100 KHz. In applications of this type this rate is not

preferable, especially since the Nyquist limit is a theoretical limit and it is always better to over sample the signal. This means working at a maximum cross-correlation frequency of a fraction of one hertz, which is quite difficult to attain. Normally, the excitation light is modulated at a given frequency,  $f_1$ . Typically this is in the megahertz region. The fluorescence will also be modulated at this frequency, but the signal will be phase-shifted and demodulated with respect to the excitation light. The fluorescence light is detected with an intensifier which is modulated at  $f_1$ , plus a small frequency,  $f_c$ . The two frequencies,  $f_1$  from fluorescence and  $f_1 + f_c$  from the modulator, will mix to produce a signal at  $f_c$  with the same phase and demodulation information as the fluorescence. This frequency translation is called heterodyning. Adding another modulator or mechanical chopper, which is phase locked to the modulation frequency of the intensifier, to modulate the excitation or emission light at the cross-correlation frequency,  $f_c$  is a solution to this problem. This additional signal mixes with the heterodyned signal inside the intensifier. The result is to frequency translate the heterodyned signal which is at a frequency,  $f_c$ , to DC. This is called homodyning. The DC signal is then detected by the diode array, or some other parallel array detector to give one point of the low frequency sine wave. The phase of the chopper modulator, is then shifted by a small amount. The amount depends on the number of points to be collected in a full wave which is 360 degrees. If 16 points per wave are to be collected, the phase must be shifted by 22.5 degrees for each run, and the DC is collected. This process is repeated until the phase is shifted a full 360 degrees. The result is a series of points that map the sinusoidal wave from which the phase and demodulation of the fluorescence signal can be derived.

Referring to Figure 2a, the parallel phase fluorometer utilizing the detection and cross-correlation means described in conjunction with Figure 2 is shown incorporating the improved parallel array detector. The chopper modulator 200 is in line between the sample holder 64 and the polychromator 64. The chopper modulator 200 receives a frequency synchronizing signal from the frequency synthesizer 57 in order to be phase locked to the modulation frequency of the intensifier 70. The chopper modulator 200 is a simple mechanical device in which rotating fins or blades allow light to pass through at a predetermined frequency based on the speed of rotation. As was indicated above, the speed of rotation is set by the frequency synchronizing signal from the frequency synthesizer 57.

There are many advantages to the above described technique. The most important is that this method makes it possible to use much higher cross-correlation frequencies. It is expensive to buy frequency generators that can operate at approximately 300 MHz with a resolution of a fraction of one hertz. The system is simplified because no new exotic parts are needed. Since the output signal is DC, the speed of the phosphor and the read rate of the detector become unimportant. A second important advantage is the use of a two-stage down-conversion process, instead of one-stage down-conversion. Theoretically, this type of measurement can be done in one stage. The intensifier can be modulated at the same frequency as the fluorescence. The result would be a DC that would vary with the phase difference between the phase of the signal sent to modulate the excitation light and the phase of the signal sent to the intensifier. However, in practice, it is very difficult to set a phase difference at 100 MHz or so to an accuracy of less than one degree. This method avoids such problems by doing the homodyning at the much lower cross-correlation frequency,  $f_c$ .

As is stated above, the array detector is not  
limited to use in fluorescence measurement systems. The  
1 array detector can be utilized in systems that require light  
modulation or light heterodyning techniques. In these  
applications, fluorescence measurements are not taken, but  
rather modulated light which has been utilized in any number  
5 of ways.

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WHAT IS CLAIMED:

1. A system for measuring the light emitted by a sample when excited by a light source, said system having an array detector for detecting the modulated light emissions of an excited sample, said array detector comprising:

(a) a semiconductor array for receiving and recording the level of modulated light emissions received from said sample;

(b) an image intensifier positioned between said sample and said semiconductor array, said image intensifier modulating the gain of the modulated light emissions received from said sample and translating said emissions from a first frequency range to a second frequency range;

(c) means for selectively biasing said image intensifier to effectively pass to said array detector emissions having frequencies of  $f_1$  and  $f_c$ ; and

(d) means for reading at a frequency other than  $f_c$ , the recorded levels of modulated light emissions of said array detector.

2. The array detector as claimed in Claim 1 further comprising means for coupling said semiconductor array with said image intensifier, said array detector translates said modulated light emissions into electrical signals while maintaining a spatial geometry equivalent to said modulated light emissions.

3. The array detector as claimed in Claim 2 wherein said spatial geometry is maintained on a pixel by pixel geometry in an X-Y plane thus allowing the emissions from said sample to be studied at discrete X-Y locations.

4. The array detector as claimed in Claim 3 wherein said coupling means comprises an optical fiber coupler.

5. The array detector as claimed in Claim 4 wherein said semiconductor array is a charged-coupled device.

6. The array detector as claimed in Claim 4 wherein said semiconductor array is a diode array.

7. The array detector as claimed in Claim 1  
wherein said image intensifier translates the modulated light  
1 emission received from said sample from said first frequency  
range to said second frequency range by modulating the gain  
of said emissions utilizing a radio frequency modulating  
signal.

5 8. The array detector as claimed in Claim 7  
wherein said first frequency range is the mega hertz  
frequency range and said second frequency range is the hertz  
frequency range.

10 9. The array detector as claimed in Claim 8  
wherein said modulation is a heterodyning technique for the  
frequency translation of said modulating light emissions  
received from said sample to a frequency range for display  
and recording by said array detector.

15 10. The array detector as claimed in Claim 9  
wherein said means for selectively biasing said image  
intensifier is a circuit for adjusting the modulation gain of  
said image intensifier.

20 11. A system for measuring the light emitted by a  
sample when excited by a light source, said system having an  
array detector for detecting the modulated light emissions of  
an excited sample, said array detector comprising:

(a) a semiconductor array for receiving and  
recording the level of modulated light emissions received  
from said sample;

25 (b) an image intensifier positioned between said  
sample and said semiconductor array, said image intensifier  
modulating the gain of the modulated light emissions from a  
first frequency range to a second frequency range;

30 (c) means for selectively biasing said image  
intensifier to effectively pass to said array detector  
emissions having frequencies of  $f_1$  and  $f_c$ ;

(d) a modulator means for further modulating said  
modulated light emissions from said sample at said frequency  
 $f_c$ ; and

35 (e) means for reading at a frequency other than  
 $f_c$ , the recorded levels of modulated light emissions of said  
array detector.

12. The array detector as claimed in Claim 11 further comprising means for coupling said semiconductor  
1 array with said image intensifier, said array detector translates said modulated light emissions into electrical signals while maintaining a spatial geometry equivalent to said modulated light emissions.

5 13. The array detector as claimed in Claim 12 wherein said spatial geometry is maintained on a pixel by pixel geometry in an X-Y plane thus allowing the emissions from said sample to be studied at discrete X-Y locations.

10 14. The array detector as claimed in Claim 11 wherein said image intensifier translates the modulated light emission received from said sample from said first frequency range to said second frequency range by modulating the gain of said emissions utilizing a radio frequency modulating signal.

15 15. The array detector as claimed in Claim 14 wherein said first frequency range is the mega hertz frequency range and said second frequency range is the hertz frequency range.

20 16. The array detector as claimed in Claim 15 wherein said modulation is a heterodyning technique for the frequency translation of said modulating light emissions received from said sample to a frequency range for display and recording by said array detector.

25 17. The array detector as claimed in Claim 16 wherein said heterodyning technique utilizes a unique gating technique which cross-correlates a high frequency source at  $f_1$  with said image intensifier at a frequency of  $f_1 + f_c$  to optically cross-correlate the fluorescence emission response phase and modulation information onto  $f_c$ , resulting in a  
30 heterodyned signal.

35 18 The array detector as claimed in Claim 17 wherein said modulator means is a mechanical chopper phase locked with the modulation frequency of said intensifier, a modulated output from said mechanical chopper is mixed with said heterodyned signal resulting in a DC signal.

19. The array detector as claimed in Claim 18 wherein said semiconductor array detects said DC signal to  
1 record one point of a low frequency sine wave from which phase and demodulation of the emitted signal is derived.

20. The array detector as claimed in Claim 18 wherein said mechanical chopper is phase shifted a  
5 predetermined number of times in order to collect a series of points of said low frequency sine wave from which phase and demodulation of the emitted signal can be derived.

21. The array detector as claimed in Claim 11 wherein said means for selectively biasing said image  
10 intensifier is a circuit for adjusting the modulation gain of said image intensifier.

22. An array detector for detecting the modulated light emissions of an excited sample, said array detector comprising:  
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(a) a semiconductor array for receiving and recording the level of modulated light emissions received from said sample;

(b) an image intensifier positioned between said sample and said semiconductor array, said image intensifier  
20 modulating the gain of the modulated light emissions from a first frequency range to a second frequency range;

(c) means for selectively biasing said image intensifier to effectively pass to said array detector emissions having frequencies of  $f_1$  and  $f_c$ ;  
25

(d) a modulator means for further modulating said modulated light emissions from said sample at said frequency  $f_c$ ; and

(e) means for reading at a frequency other than  $f_c$ , the recorded levels of modulated light emissions of said  
30 array detector.

23. The array detector as claimed in Claim 22 further comprising means for coupling said semiconductor array with said image intensifier, said array detector translates said modulated light emissions into electrical  
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signals while maintaining a spatial geometry equivalent to said modulated light emissions, said spatial geometry is maintained on a pixel by pixel geometry in an X-Y plane thus allowing the emissions from said sample to be studied at discrete X-Y locations.

24. The array detector as claimed in Claim 23 wherein said image intensifier translates the modulated light emission received from said sample from the mega hertz frequency range to the hertz frequency range by modulating the gain of said emissions utilizing a radio frequency modulating signal, said modulation is a heterodyning technique for the frequency translation of said modulating light emissions received from said sample to a frequency range for display and recording by said array detector, said heterodyning technique utilizes a unique gating technique which cross correlates a high frequency source at  $f_1 + f_c$  to optically cross-correlate the fluorescence emission response phase and modulation information onto  $f_c$ , resulting in an heterodyned signal.

25. The array detector as claimed in Claim 24 wherein said modulator means is a mechanical chopper phase locked with the modulation frequency of said intensifier, a modulated output from said mechanical chopper is mixed with said heterodyned signal resulting in a DC signal, said semiconductor array detects said DC signal to record one point of a low frequency sine wave from which phase and demodulation of the emitted signal is derived, said mechanical chopper is phase shifted a predetermined number of times in order to collect a series of points of said low frequency sine wave from which phase and demodulation of the emitted signal can be derived.

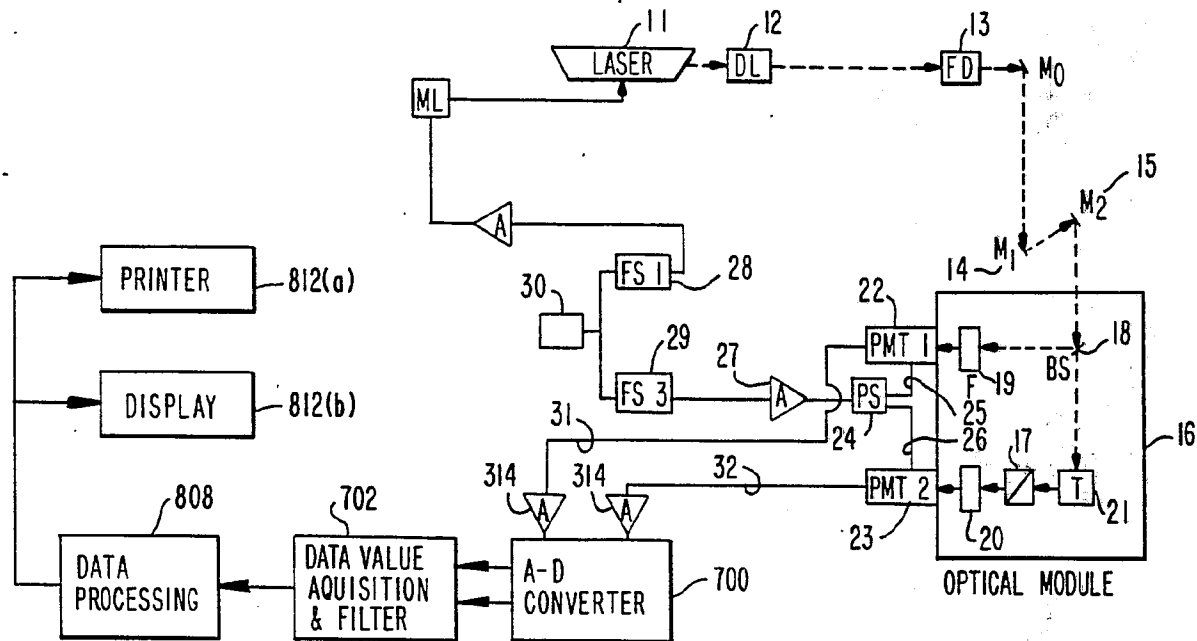
$\frac{1}{6}$ 

FIG. 1

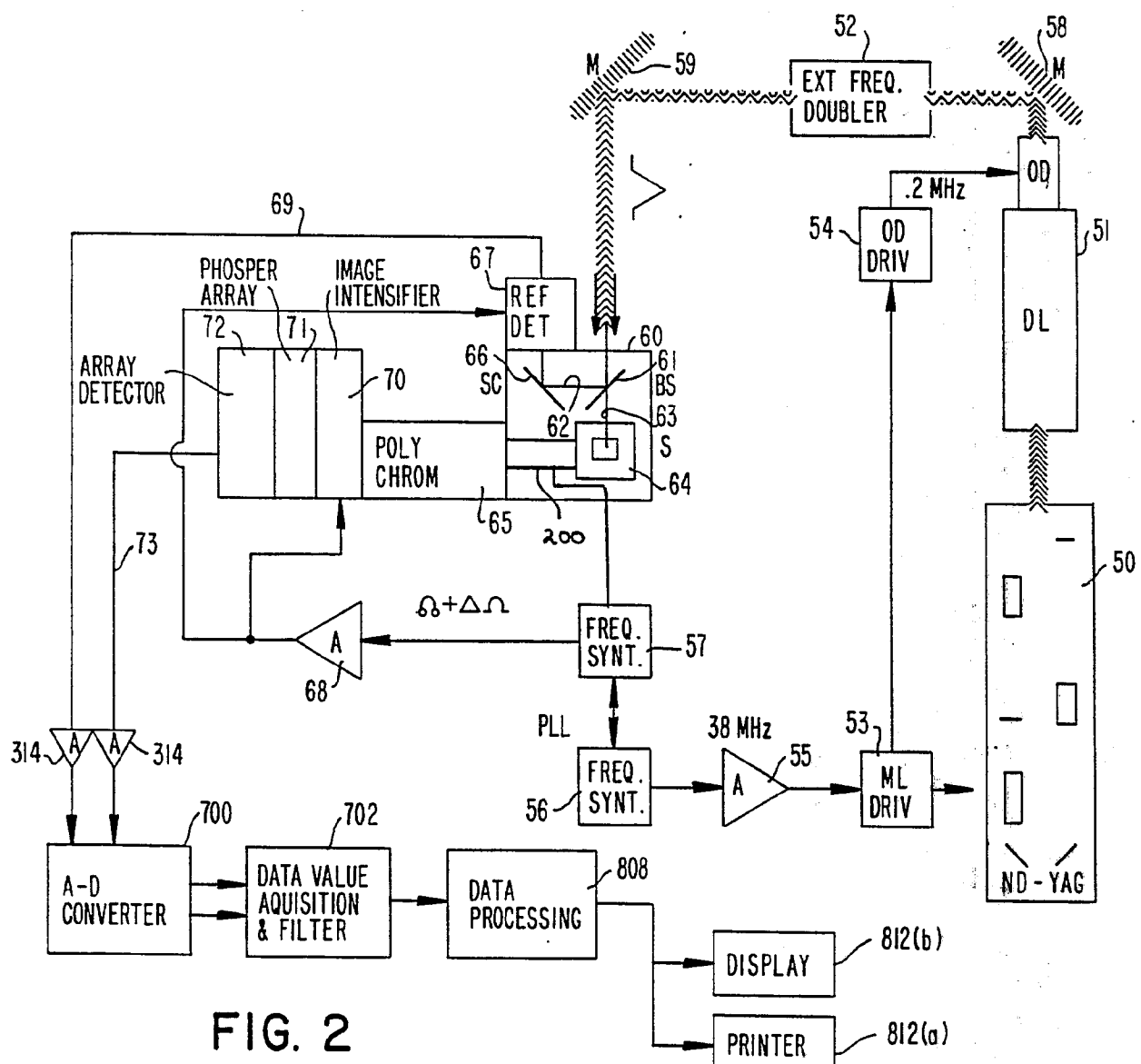


FIG. 2

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FIG. 3

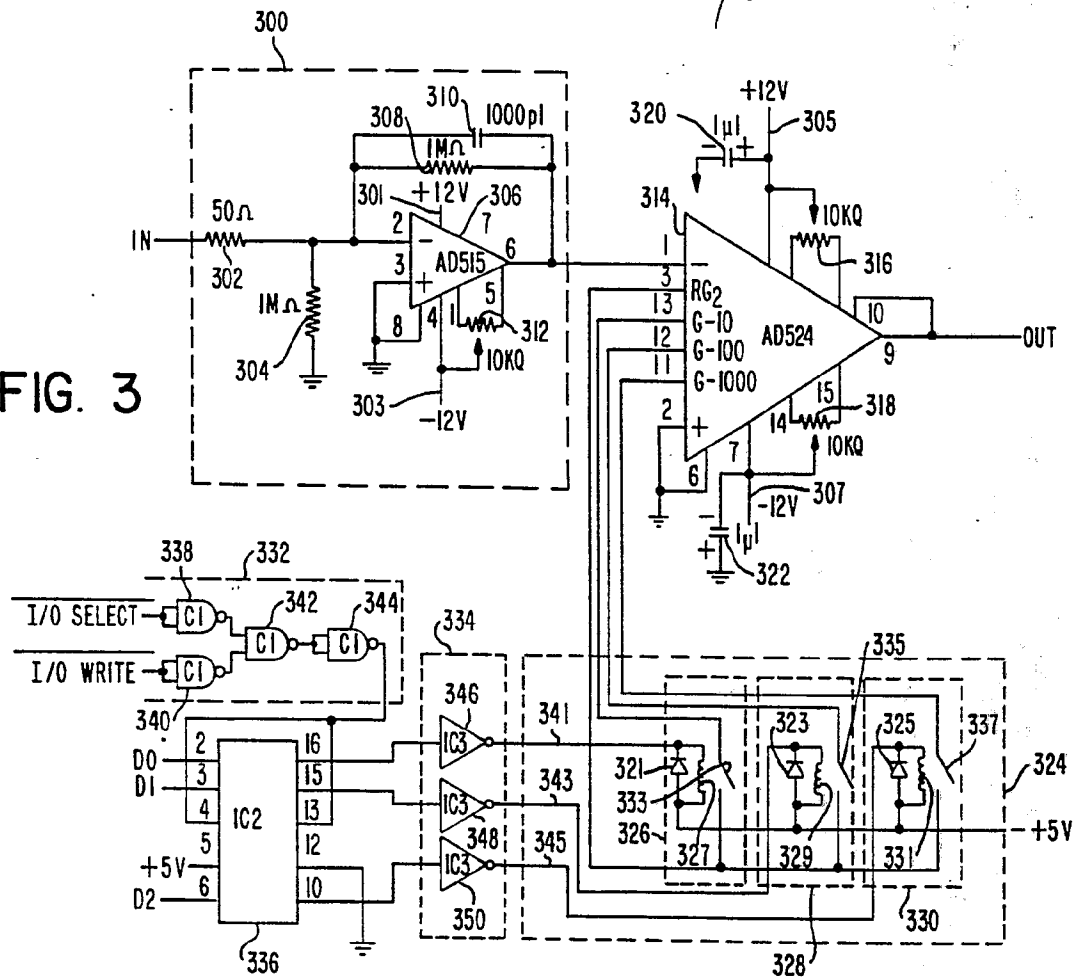
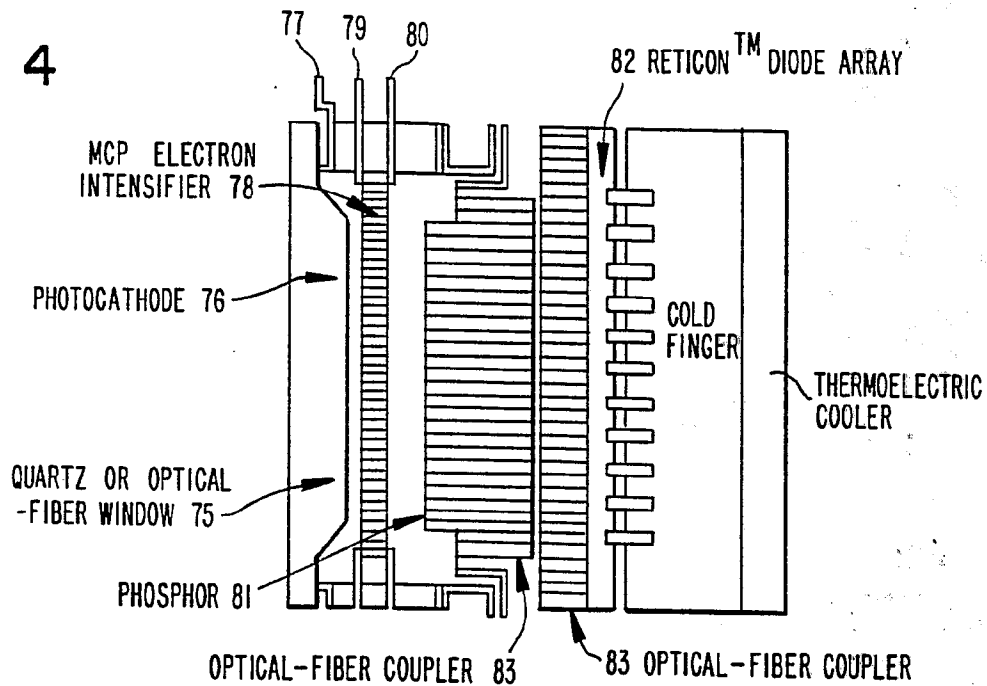


FIG. 4



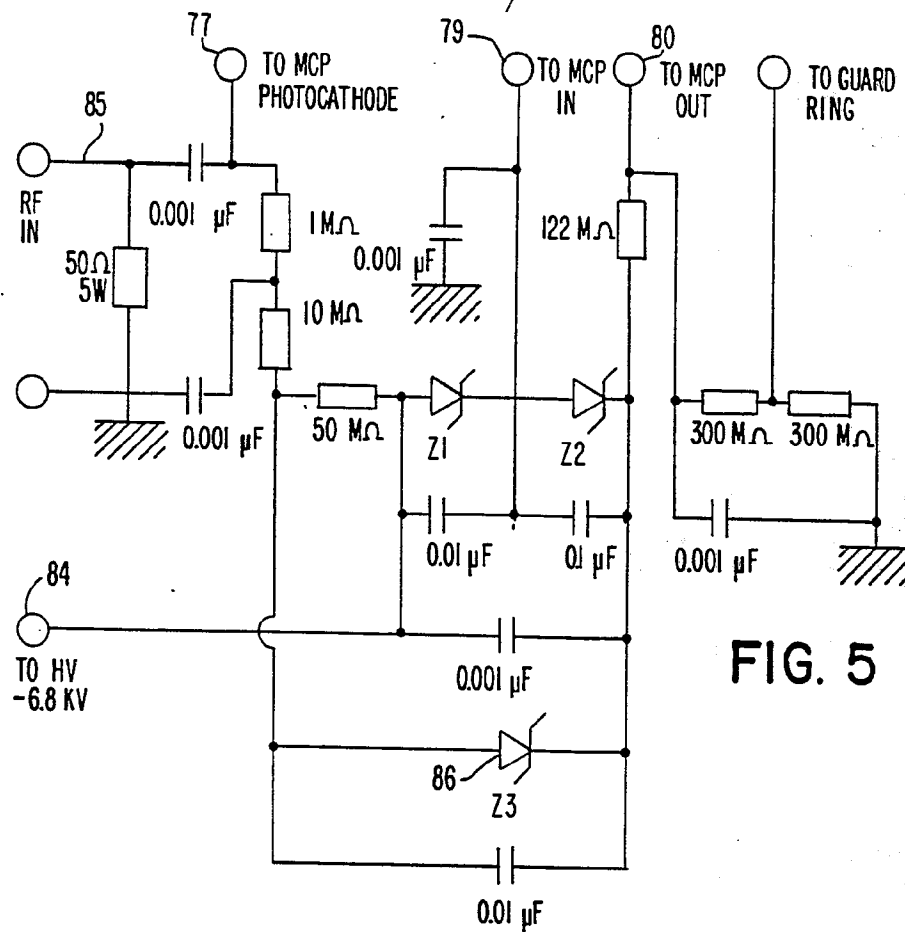
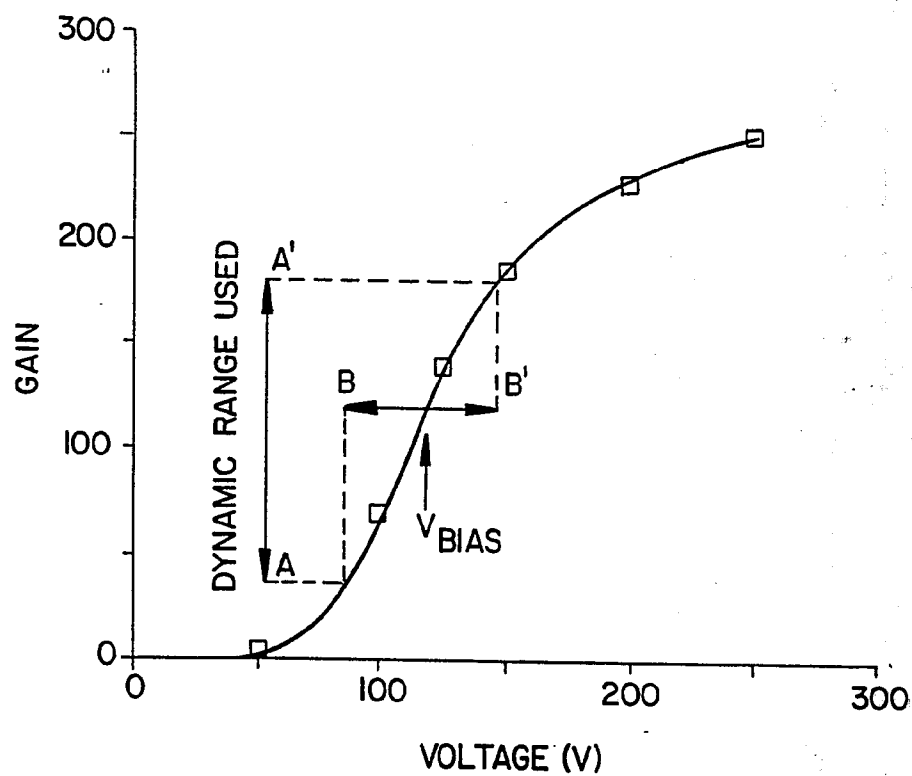
$$\frac{3}{6}$$


FIG. 5

FIG. 6





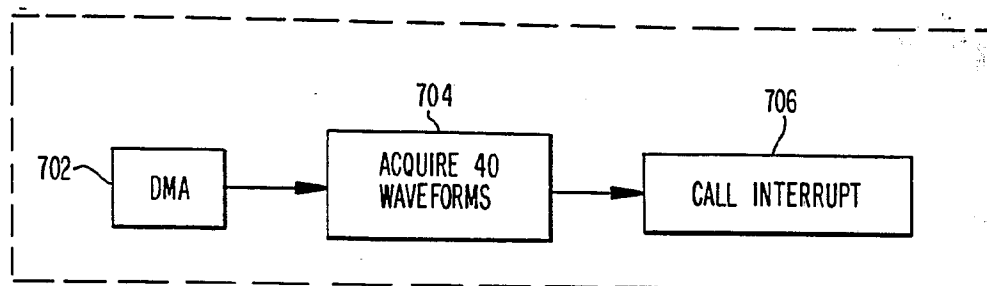


FIG. 7

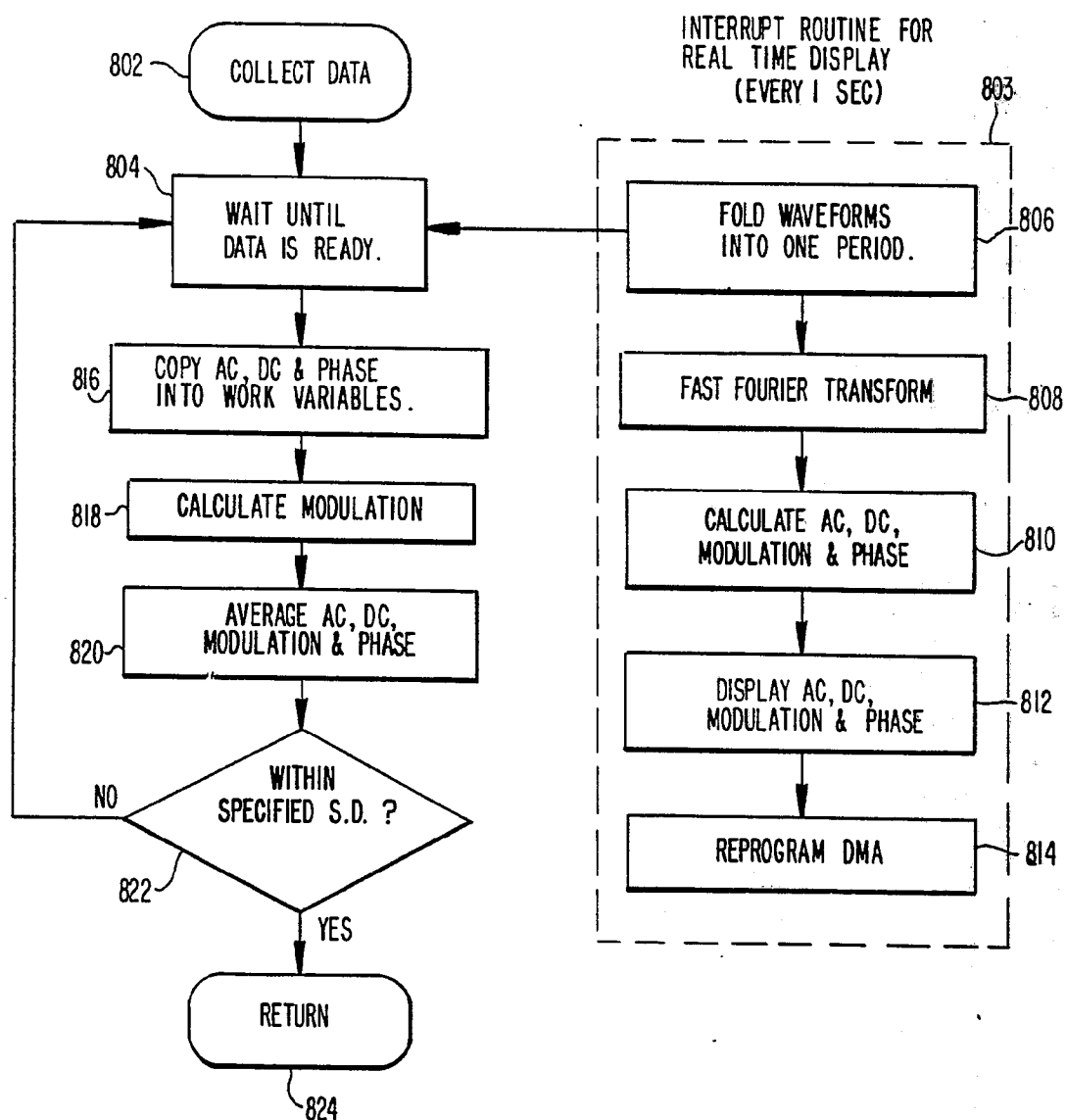
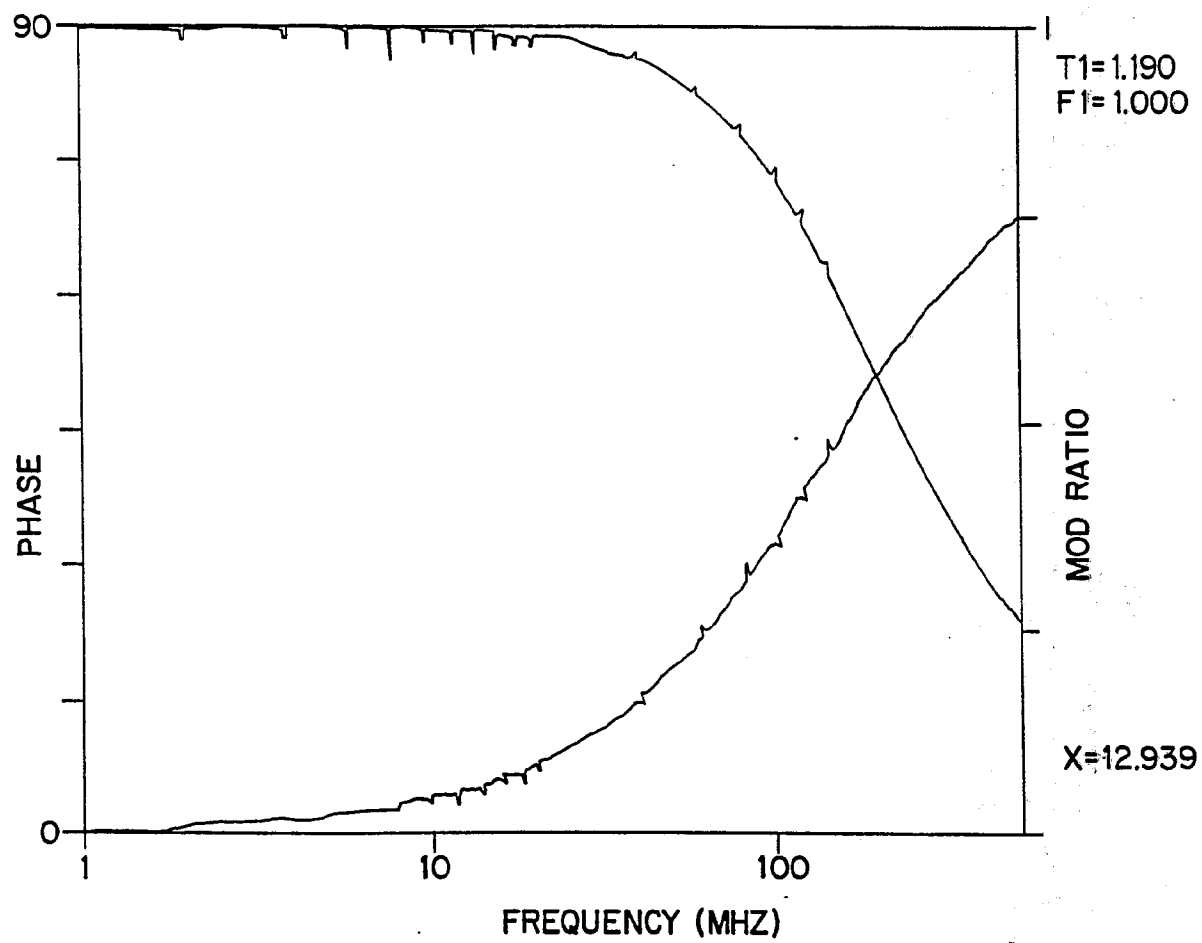


FIG. 8

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FIG. 9



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FIG. 10A

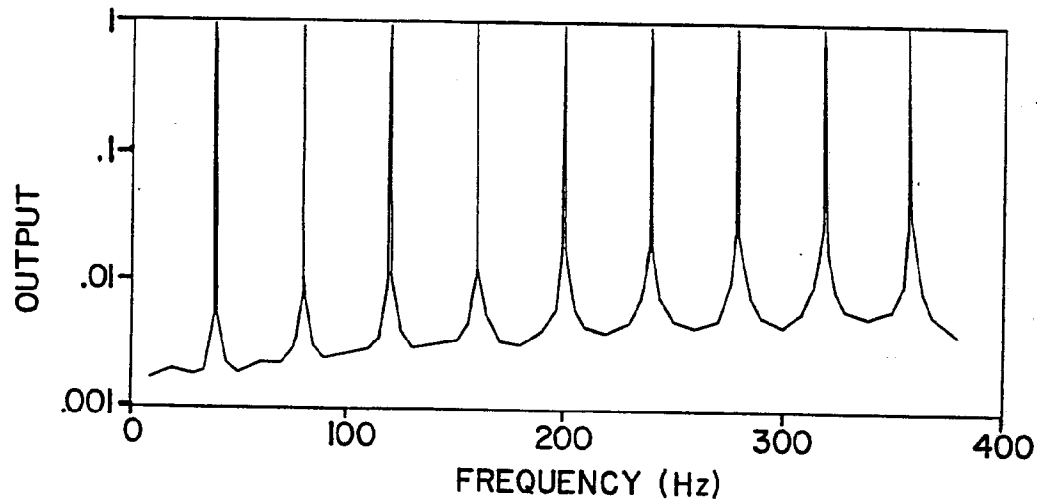


FIG. 10B

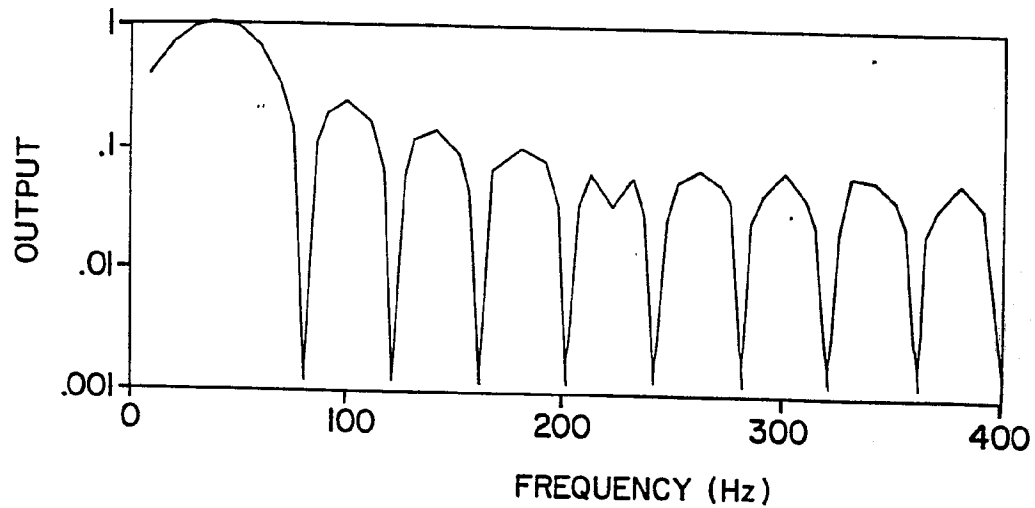
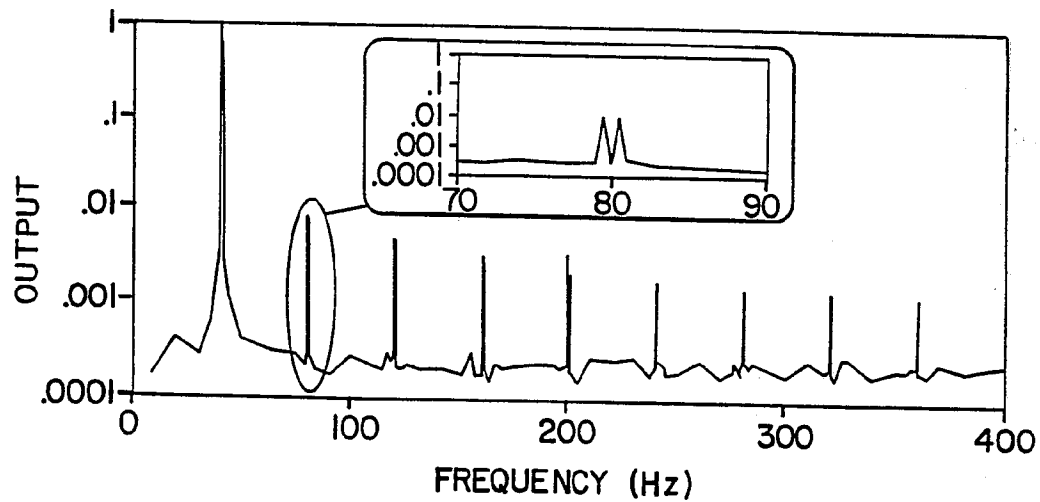


FIG. 10C



# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/00818

## I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): G06F 15/20,  
U S CL 364/498; 356/318,250/458.1

## II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System

Classification Symbols

U S 364/498,497,728.03, 724.06, 576,485,525  
356/317,318,300  
250/458.1, 281,288

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched

## III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of Document, <sup>1</sup> with indication, where appropriate, of the relevant passages <sup>2</sup>	Relevant to Claim No. <sup>3</sup>
A	US, A, 4,421,860 (ELINGS ET AL) 20 December 1983 see abstract	1-25
A	US, A, 3,973,112 (SLOANE) 03 August 1976 see abstract	1-25
A	US, A, 4,582,809 (BLOCK ET AL) 15 April 1986 see abstract	1-25
A	US, A, 4,100,416 (HIRSCHFELD) 11 July 1978 see abstract	1-25

### \* Special categories of cited documents: <sup>10</sup>

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

Date of Mailing of this International Search Report

27 April 1990

**21 MAY 1990**

International Searching Authority

Signature of Authorized Officer

ISA/US

Ellis B. Ramirez